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Inventor(s): BOUDEC PHILIPPE;; BOURDON HELENE;; DUMAS FLORENCE;; SAILLAND ALAIN

Applicant(s): RHONE POULENC AGROCHIMIE (FR)

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Abstract

The invention concerns a chimeric hydroxy-phenyl pyruvate dioxygenase (HPPD), comprising the N-terminal part of a first HPPD associated with the C-terminal part of a second HPPD, a nucleic acid sequence coding for said chimera HPPD, a chimeric gene containing said sequence as coding sequence and its use for obtaining plants resistant to certain herbicides.

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(71) Déposant: ~~RHONE POULENC AGRO~~ [FR/FR]; ~~14-20, rue Pierre Baizet, F-69009 Lyon (FR)~~. 55 avenue Rene Cassin F-69009 Lyon

(72) Inventeurs: BOUDEC, Philippe; 7, rue Camille de Neuville, F-69009 Lyon (FR). BOURDON, Hélène; 8, allée des Tullistes, F-69130 Ecully (FR). DUMAS, Florence; 3, rue de la Pêcherie, F-69250 Fleurieu sur Saône (FR). RODGERS, Matthew; Rhône-Poulenc Agriculture Ltd., Fyfield Road, Ongar, Essex CM5 0HW (GB). SAILLAND, Alain; 38, rue Albert Chalinel, F-69009 Lyon (FR).

(74) Mandataire: TETAZ, Franck; Rhône-Poulenc Agro, Boîte postale 9163, F-69263 Lyon Cedex 09 (FR).

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(54) Titre: HYDROXY-PHENYL PYRUVATE DIOXYGENASE MUTEE, SEQUENCE D'ADN ET OBTENTION DE PLANTES CONTENANT UN TEL GENE, TOLERANTES AUX HERBICIDES

(57) Abstract

The invention concerns a nucleic acid sequence coding for a mutated hydroxy-phenyl pyruvate dioxygenase (HPPD) with improved tolerance to HPPD inhibitors, a chimera gene containing said sequence as coding sequence and its use for obtaining plants resistant to certain herbicides.

(57) Abrégé

La présente invention concerne une séquence d'acide nucléique codant pour une hydroxy-phényl pyruvate dioxygénase (HPPD) mutée présentant une tolérance améliorée aux inhibiteurs d'HPPD, un gène chimère contenant cette séquence comme séquence codante et son utilisation pour l'obtention de plantes résistantes à certains herbicides.

Mutated hydroxyphenylpyruvate dioxygenase, DNA sequence and isolation of plants which contain such a gene and which are tolerant to herbicides.

The present invention relates to a nucleic acid sequence encoding a mutated hydroxyphenylpyruvate dioxygenase (HPPD), to a chimeric gene which comprises this sequence as the coding sequence, and to its use for isolating plants which are resistant to certain herbicides.

The hydroxyphenylpyruvate dioxygenases are enzymes which catalyse the reaction in which para-hydroxyphenylpyruvate (HPP) is transformed into homogentisate. This reaction takes place in the presence of iron (Fe^{2+}) and in the presence of oxygen (Crouch N.P. et al., Tetrahedron, 53, 20, 6993-7010, 1997). It may be hypothesized that the HPPDs contain an active site which is capable of catalysing this reaction, in which iron, the substrate and the molecule of water link together, although such an active site has not so far been described.

Some molecules which inhibit this enzyme, and which bind to the enzyme competitively in order to inhibit transformation of the HPP into homogentisate, are also known. Some of these molecules have been used as herbicides since inhibition of the reaction in plants leads to whitening of the leaves of the treated plants and to the death of the said plants

(Pallett K.E. et al. 1997 Pestic. Sci. 50 83-84). The herbicides for which HPPD is the target, and which are described in the state of the art, are, in particular, isoxazoles (EP 418 175, EP 470 856, EP 487 352, EP 527 036, EP 560 482, EP 682 659, US 5 424 276), in particular isoxaflutole, which is a selective herbicide for maize, diketonitriles (EP 496 630, EP 496 631), in particular 2-cyano-3-cyclopropyl-1-(2-SO_2CH_3-4-CF_3 phenyl)propane-1,3-dione and 2-cyano-3-cyclopropyl-1-(2-SO_2CH_3-4-2,3Cl_2phenyl)propane-1,3-dione, triketones (EP 625 505, EP 625 508, US 5,506,195), in particular sulcotrione, or else pyrazolinates.

Three main strategies are available for making plants tolerant to herbicides, i.e. (1) detoxifying the herbicide with an enzyme which transforms the herbicide, or its active metabolite, into non-toxic degradation products, such as, for example, the enzymes for tolerance to bromoxynil or to basta (EP 242 236, EP 337 899); (2) mutating the target enzyme into a functional enzyme which is less sensitive to the herbicide, or to its active metabolite, such as, for example, the enzymes for tolerance to glyphosate (EP 293 356, Padgette S.R. et al., J. Biol. Chem., 266, 33, 1991); or (3) overexpressing the sensitive enzyme so as to produce quantities of the target enzyme in the plant which are sufficient in relation to the herbicide, in view of the kinetic constants of this

enzyme, so as to have enough of the functional enzyme available despite the presence of its inhibitor.

It is this third strategy which was described for successfully obtaining plants which were tolerant to HPPD inhibitors (WO 96/38567), with it being understood that this was the first time that a strategy of simply overexpressing the (non-mutated) sensitive target enzyme was successfully used for conferring on plants agricultural level tolerance to a herbicide.

Despite the success obtained with this strategy of simply overexpressing the target enzyme, it is still necessary to improve the system of tolerance to HPPD inhibitors in order to obtain a tolerance whatever the conditions under which the tolerant plants are cultivated, or the commercial doses at which the herbicides are applied in the fields, may be.

The present invention therefore relates, first and foremost, to a mutated HPPD which, while being functional, that is to say while retaining its properties of catalysing the transformation of HPP into homogentisate, is less sensitive to HPPD inhibitors than is the native HPPD before mutation.

In view of the competitive character of the inhibition, it may be hypothesized that the HPPD inhibitors bind to the enzyme in its active site, or in the vicinity of this active site, so as to block access of the HPP to this active site and prevent its transformation in the presence of iron and oxygen. By

effecting a mutation which limits the access of the inhibitor to the active site of the enzyme, while at the same time safeguarding access of the HPP to the active site, it is possible to obtain functional mutated enzymes which are less sensitive to HPPD inhibitors.

It was then observed that, by mutating the enzyme in its C-terminal part, it was possible to obtain functional HPPDs which were less sensitive to HPPD inhibitors, such that expression of these functional HPPDs in plants improves the tolerance of the plants to HPPD inhibitors.

The present invention therefore relates to a novel functional mutated HPPD which is less sensitive to HPPD inhibitors and which contains at least one mutation in its C-terminal part.

According to the invention, "mutation" is understood as being the replacement of an amino acid of the primary sequence with another amino acid. The expression "mutated amino acid" will be used below to designate the amino acid which is replaced by another amino acid, thereby designating the site of the mutation in the primary sequence of the protein.

Several HPPDs and their primary sequences have been described in the state of the art, in particular the HPPDs of bacteria such as *Pseudomonas* (Rüetschi et al., Eur. J. Biochem., 205, 459-466, 1992, WO 96/38567), of plants such as *Arabidopsis*

(WO 96/38567, Genebank AF047834) or of carrot (WO 96/38567, Genebank 87257) of *Coccicoides* (Genebank COITRP) or of mammals such as the mouse or the pig.

By aligning these known sequences, by using the customary means of the art, such as, for example, the method described by Thompson, J.D. et al. (CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22; 4673-4680, 1994), and accessing these computer programs for sequence alignment which are accessible via the Internet, for example, the skilled person is able to define the sequence homologies in relation to a reference sequence and find the key amino acids or else define common regions, making it possible, in particular, to define a C-terminal region and an N-terminal region on the basis of this reference sequence.

In the case of the present invention, the reference sequence is the *Pseudomonas* sequence, with all the definitions and indications of the positions of particular amino acids being made with respect to the primary *Pseudomonas* HPPD sequence. The attached Figure 1 depicts an alignment of several HPPD sequences which are described in the state of the art; these sequences are aligned with respect to the *Pseudomonas* HPPD sequence as the reference sequence and comprise the HPPD sequences of *Streptomyces avermitilis*

(Genebank SAV11864), of *Daucus carota* (Genebank DCU 87257), of *Arabidopsis thaliana* (Genebank AF047834), of *Zea mais*, of *Hordeum vulgare* (Genebank HVAJ693), of *Mycosphaerella graminicola* (Genebank AF038152), of *Coccicoides immitis* (Genebank COITRP) and of *Mus musculus* (Genebank MU54HD). This figure gives the numbering of the amino acids of the *Pseudomonas* sequence and also the amino acids which are common to these sequences, with these amino acids being designated by an asterisk. On the basis of such an alignment, it is easy, from the definition of the *Pseudomonas* amino acid by its position and its nature, to identify the position of the corresponding amino acid in another HPPD sequence (with the alignment of sequences of different plant, mammalian and bacterial origin demonstrating that this method of alignment, which is well known to a skilled person, can be generalized to any other sequence). An alignment of different HPPD sequences is also described in Patent Application WO 97/49816.

The C-terminal part of the HPPDs, which is where the active site of the enzyme is located, differs from its N-terminal part by a linking peptide which ensures the stability of the enzyme and its oligomerization (the *Pseudomonas* HPPD is a tetramer while the plant HPPDs are dimers), as the diagrammatic depiction of the tertiary structure of the *Pseudomonas* HPPD monomer shown in Figure 2 demonstrates. This

structure was obtained by the customary methods of studying crystal X-ray diffraction. The linking peptide makes it possible to define the N-terminal end of the C-terminal part of the enzyme, with the said peptide being located between amino acids 145 and 157 in the case of *Pseudomonas* (cf. Figure 1).

The C-terminal part can therefore be defined as consisting of the sequence defined, on the one hand, by the linking peptide and, on the other hand, by the C-terminal end of the enzyme, with the mutation which is effected in the C-terminal part of the HPPD therefore being effected in the region which has thus been defined. Two amino acids, which are in positions 161 and 162 in the case of the *Pseudomonas* sequence (D = Asp161 and H = His162), will be noted in all sequences shown in the sequence alignment depicted in the attached Figure 1. With reference to the *Pseudomonas* HPPD, it is therefore possible to define the linking peptide which represents the N-terminal end of the C-terminal part of the HPPD as being located between approximately 5 and 15 amino acids upstream of the amino acid Asp161.

According to a preferred embodiment of the invention, the mutation is effected on amino acids which are replaced with amino acids exhibiting greater steric hindrance or else with one of the ionized or ionizable amino acids. Preferably, the mutation is effected on amino acids which have low steric

hindrance. According to the invention, an amino acid of low steric hindrance is preferably understood as being glycine or amino acids with low steric hindrance such as alanine, cystein, serine, etc.

Any amino acid which exhibits greater steric hindrance than the replaced amino acid can be employed for the mutation according to the invention.
Preferably, the amino acids of the mutation site are replaced with the following amino acids: leucine, isoleucine or tryptophan.

According to the invention, an ionized or ionizable amino acid is understood as being any amino acid which exhibits, in addition to the groups which enter into the peptide bond, an amino, carboxylic acid (COOH) or ammonium or $-COO^-$ group. The following amino acids of this nature are preferred: glutamine and glutamic acid or asparagine or aspartic acid.

According to a preferred embodiment of the invention, the mutation is effected on an amino acid of the C-terminal part which is common to several HPPD sequences, with it being possible to identify these latter by the sequence alignment method.

According to a particular embodiment of the invention, the mutated HPPD contains, in its C-terminal part, the following peptide sequence:

- Gly - Phe - Xaa - Yaa - Xab - Asn - Phe - Yab - Yac - Leu - Phe -

in which Xaa and Xab, independently of each other represent glycine (Gly) or an amino acid which exhibits a hindrance which is greater than that of glycine, with it being understood that if either Xaa or Xab represents Gly, the other amino acid is then different from Gly,

Yaa represents any amino acid, preferably Ala, Lys or Glu*,

Yab represents any amino acid, preferably Lys, Ser, Arg or Asn*, and

Yac represents any amino acid, preferably Ala, Ser, Glu or Gln*.

Advantageously, at least one of Xaa and Xab represents Leu, Glu, Trp or Ile.

With reference to the *Pseudomonas* HPPD sequence, the mutated amino acids are selected from the following amino acids: Pro215, Gly298, Gly332, Phe333, Gly334, Gly336 and Asn 337, more preferably the amino acids Pro215 and Gly336.

The following preferred examples of mutations may be cited: Pro215Leu, Gly336Glu, Gly336Trp or Gly336Ile.

It is understood that the above-described mutations can be combined in pairs, such as, for example, a double mutation of the amino acids Gly334 and Gly336.

The present invention also relates to a nucleic acid sequence which encodes a mutated HPPD as described

above. According to the present invention, a "nucleic acid sequence" is understood as being a nucleotide sequence which can be of the DNA or RNA type, preferably of the DNA type, and in particular double-stranded, whether it be of natural or synthetic origin, in particular a DNA sequence in which the codons which encode the mutated HPPD according to the invention will have been optimized in accordance with the host organism in which it is to be expressed, with these methods of optimization being well known to the skilled person.

The sequence which encodes an original unmutated HPPD can be of any origin whatever. In particular, it can be of bacterial origin. Advantageous examples which may be cited are bacteria of the *Pseudomonas sp.* type, for example *Pseudomonas fluorescens*, or else cyanobacteria of the *Synechocystis* type. The sequence can also be of plant origin, in particular derived from dicotyledonous plants such as tobacco, *Arabidopsis*, umbelliferous plants such as *Daucus carotta*, or else monocotyledonous plants such as *Zea mais* or wheat or else barley. The coding sequences, and the way of isolating and cloning them, are described in the previously cited references, the contents of which are hereby incorporated by reference.

The mutation can be effected in the nucleic acid sequence which encodes the original unmutated HPPD by any means which is appropriate for replacing, in the

said sequence, the codon which encodes the mutated amino acid with the codon which corresponds to the amino acid which is to replace it, with the said codons being widely described in the literature and well known to the skilled person.

Several molecular biological methods can be used to achieve this mutation.

A first method consists in subjecting cell cultures to long-term selection pressure with an inhibitor of the HPPD, in the presence or absence of a mutagenic agent, with the HPPD gene then mutating spontaneously under the effect of this selection pressure and, where appropriate, the mutagenic agent, with the said gene having changed such that it encodes a mutated enzyme which enables HPPD activity to be expressed under conditions under which the unmodified enzyme is partially or totally inhibited. The cells can be plant cells or bacteria and, in this latter case, they can express a native HPPD (of bacterial origin) or an HPPD of another origin (bacterial, fungal, algal or plant) which has been introduced into the bacterium employed for the mutagenesis in an appropriate form which permits expression of this HPPD, with the gene encoding the native HPPD of the said bacterium having preferably been deleted, if it exists. Such methods of transforming bacteria are well known to the skilled person, and are amply described in the literature, as are the methods of mutation (in particular: Sambrook

et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

When the cell is a plant cell which is expressing a native HPPD, the mutated HPPD can be isolated and cloned or else plants can be regenerated from the cell cultures using the customary methods. The resulting plants then express a functional mutated HPPD which is less sensitive to HPPD inhibitors than is the native HPPD. The methods of regeneration are amply described in the literature (including the previously cited references) and well known to the skilled person.

The cells which exhibit a mutated HPPD which is less sensitive to HPPD inhibitors are selected using an appropriate screening aid. In view of the object of the present invention, and the sought-after solution, i.e. an HPPD which is less sensitive to HPPD inhibitors, a screening aid which is simple to implement consists in determining the doses of HPPD inhibitor which fully inhibit the original unmutated HPPD, and which are lethal for the cells which express this unmutated HPPD, in subjecting the cells, after mutation, to this predetermined dose, in isolating the mutated cells which have withstood this lethal dose, and then isolating and cloning the gene which encodes the mutated HPPD.

This process of mutagenesis by cell culture was carried out on a very substantial number of

Pseudomonas cells which were expressing their native HPPD (see, in particular, the specific examples described below). In all cases, the mutants which were isolated by the above-defined selection method were mutants which exhibited a mutation in the C-terminal part of the HPPD.

Another method for preparing a mutated nucleic acid sequence according to the invention, and the corresponding protein, consists in carrying out site-directed mutagenesis on one or more amino acids which are selected in advance, for example by identifying the amino acids which are common to several sequences in the C-terminal part, or else by attempting to reproduce, in an HPPD of one specific origin, a mutation which was obtained by random mutagenesis (cell culture) in an HPPD of another origin. The methods for obtaining these site-directed mutations are well known to the skilled person and widely described in the literature (in particular: Directed Mutagenesis: A Practical Approach, 1991, Edited by M.J. McPHERSON, IRL PRESS), or are methods for which it is possible to employ commercial kits (for example the U.S.E. mutagenesis kit from PHARMACIA). In all cases, it is useful, after this site-directed mutagenesis, to employ the same method as employed for the above-described random mutagenesis for selecting mutated HPPDs which are less sensitive than the corresponding unmutated HPPD.

The present invention therefore also relates to a method for preparing a nucleic acid sequence which encodes a mutated HPPD according to the invention, with the said method being defined above.

The invention also relates to the use, in a method for transforming plants, of a nucleic acid sequence which encodes a mutated HPPD according to the invention as a marker gene or as a coding sequence which makes it possible to confer on the plant tolerance to herbicides which are HPPD inhibitors. It is of course understood that this sequence can also be used in combination with (an) other gene marker(s) and/or sequence(s) which encode(s) one or more agricultural properties.

The present invention also relates to a chimeric gene (or expression cassette) which comprises a coding sequence as well as heterologous regulatory elements, in the 5' and 3' positions, which are able to function in a host organism, in particular plant cells or plants, with the coding sequence containing at least one nucleic acid sequence which encodes a mutated HPPD as previously defined.

"Host organism" is understood as being any inferior or superior unicellular or multicellular organism into which the chimeric gene according to the invention can be introduced for the purpose of producing mutated HPPD. These organisms are, in particular, bacteria, for example *E. coli*, yeasts, in

particular of the genera *Saccharomyces* or *Kluyveromyces*, *Pichia*, fungi, in particular *Aspergillus*, a baculovirus or, preferably, plant cells and plants.

"Plant cell" is understood, according to the invention, as being any cell which is derived from a plant and which is able to form undifferentiated tissues, such as calli, differentiated tissues such as embryos, parts of plants, plants or seeds.

"Plant" is understood, according to the invention, as being any differentiated multicellular organism which is capable of photosynthesis, in particular a monocotyledonous or dicotyledonous organism, more especially cultivated plants which are or are not intended for animal or human nutrition, such as maize, wheat, rape, soya bean, rice, sugar cane, beetroot, tobacco, cotton, etc.

The regulatory elements which are required for expressing the nucleic acid sequence which encodes an HPPD are well known to the skilled person and depend on the host organism. They comprise, in particular, promoter sequences, transcription activators and terminator sequences, including start and stop codons. The means and methods for identifying and selecting the regulatory elements are well known to the skilled person and widely described in the literature.

The invention relates, more especially, to the transformation of plants. Any promoter sequence of

a gene which is expressed naturally in plants, in particular a promoter which is expressed, in particular, in the leaves of plants, such as so-called constitutive promoters of bacterial, viral or plant origin, such as, for example, a histone promoter as described in application EP 0 507 698, or a promoter of rice actin or of a plant virus gene such as, for example, that of cauliflower mosaic virus (CAMV 19S or 35S), or else so-called light-dependent promoters such as that of a gene for the small subunit of plant ribulose biscarboxylase/oxygenase (RuBisCO), or any known promoter which is suitable, can be used as the promoter regulatory sequence in the plants.

According to the invention, it is also possible to use, in combination with the promoter regulatory sequence, other regulatory sequences which are located between the promoter and the coding sequence, such as transcription activators (enhancers) as, for example, the tobacco mosaic virus (TMV) translation activator which is described in application WO 87/07644 or the tobacco etch virus (TEV) activator which is described by Carrington & Freed.

Any corresponding sequence of bacterial origin, such as the nos terminator from *Agrobacterium tumefaciens*, or of plant origin, such as a histone terminator as described in application EP 0 633 317, may be used as the terminator regulatory sequence or as the polyadenylation sequence.

According to one particular embodiment of the invention, a nucleic acid sequence which encodes a transit peptide is employed 5' of the nucleic acid sequence encoding a mutated HPPD, with this transit peptide sequence being arranged between the promoter region and the sequence encoding the mutated HPPD so as to permit expression of a transit peptide/mutated HPPD fusion protein, with the mutated HPPD being previously defined. The transit peptide makes it possible to direct the mutated HPPD into the plastids, more especially the chloroplasts, with the fusion protein being cleaved between the transit peptide and the mutated HPPD as it crosses the plastid membrane. The transit peptide may be single, such as an EPSPS transit peptide (described in US patent 5,188,642) or a transit peptide of that of the plant ribulose biscarboxylase/oxygenase small subunit (RuBisCO ssu), where appropriate including a few amino acids of the N-terminal part of the mature RuBisCO ssu (EP 189 707), or else a multiple transit peptide which comprises a first plant transit peptide which is fused to a part of the N-terminal sequence of a mature protein having a plastid location, with this part in turn being fused to a second plant transit peptide as described in patent EP 508 909, and, more especially, the optimized transit peptide which comprises a transit peptide of the sunflower RuBisCO ssu fused to 22 amino acids of the N-terminal end of the maize RuBisCO ssu, in turn

fused to the transit peptide of the maize RuBisCO ssu, as described, with its coding sequence, in patent EP 508 909.

The present invention also relates to the transit peptide/mutated HPPD fusion protein, with the two elements of this fusion protein being defined above.

The present invention also relates to a cloning and/or expression vector for transforming a host organism, which vector contains at least one chimeric gene as defined above. In addition to the above chimeric gene, this vector contains at least one origin of replication. This vector can consist of a plasmid, a cosmid, a bacteriophage or a virus which has been transformed by introducing the chimeric gene according to the invention. Such transformation vectors, which depend on the host organism to be transformed, are well known to the skilled person and widely described in the literature. The transformation vector which is used, in particular, for transforming plant cells or plants is a virus, which can be employed for transforming developed plants and which additionally contains its own replication and expression elements. According to the invention, the vector for transforming plant cells or plants is preferably a plasmid.

The invention relates to a method for transforming host organisms, in particular plant cells,

by integrating at least one nucleic acid sequence or one chimeric gene as defined above, with it being possible to obtain the transformation by any appropriate known means, which means are amply described in the specialist literature and, in particular, the references cited in the present application, more especially by using the vector according to the invention.

One series of methods consists in bombarding cells, protoplasts or tissues with particles to which the DNA sequences are attached. Another series of methods consists in using, as the means for transfer into the plant, a chimeric gene which is inserted into an *Agrobacterium tumefaciens* Ti plasmid or an *Agrobacterium rhizogenes* Ri plasmid. Other methods may be used, such as microinjection or electroporation or else direct precipitation using PEG. The skilled person will choose the appropriate method depending on the nature of the host organism, in particular the plant cell or the plant.

The present invention also relates to the host organisms, in particular plant cells or plants, which are transformed and which contain a chimeric gene which comprises a sequence encoding a mutated HPPD as defined above.

The present invention also relates to the plants which contain transformed cells, in particular the plants which are regenerated from the transformed

cells. The regeneration is obtained by any appropriate method, with the method depending on the nature of the species, as described, for example, in the above references. The following patents and patent applications may be cited, in particular, with regard to the methods for transforming plant cells and regenerating plants: US 4,459,355, US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073, EP 267,159, EP 604 662, EP 672 752, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318, US 5,204,253, US 5,405,765, EP 442 174, EP 486 233, EP 486 234, EP 539 563, EP 674 725, WO 91/02071 and WO 95/06128.

The present invention also relates to the transformed plants which are derived by cultivating and/or crossing the above regenerated plants, and to the seeds of the transformed plants.

The transformed plants which can be obtained in accordance with the invention can be of the monocotyledonous type, such as cereals, sugar cane, rice and maize, or of the dicotyledonous type, such as tobacco, soya bean, rape, cotton, beetroot, clover, etc.

The invention also relates to a method for selectively weeding plants, in particular plant crops, with the aid of an HPPD inhibitor, in particular a herbicide as previously defined, which method is.

characterized in that this herbicide is applied to plants which have been transformed in accordance with the invention, either before sowing the crop, before emergence of the crop or after emergence of the crop.

The present invention also relates to a method for controlling weeds in an area of a field which contains seeds or plants which have been transformed with the chimeric gene according to the invention, which method consists in applying, to the said area of the field, a dose of an HPPD inhibitor herbicide which is toxic for the said weeds, without, however, significantly affecting the seeds or plants which have been transformed with the said chimeric gene according to the invention..

The present invention also relates to a method for cultivating the plants which have been transformed, in accordance with the invention, with a chimeric gene according to the invention, which method consists in planting the seeds of the said transformed plants in an area of a field which is appropriate for cultivating the said plants, in applying, if weeds are present, a dose, which is toxic for the weeds, of a herbicide whose target is the above-defined HPPD to the said area of the said field, without significantly affecting the said transformed seeds or the said transformed plants, and in then harvesting the cultivated plants when they reach the desired stage of

maturity and, where appropriate, in separating off the seeds of the harvested plants.

In the above two methods, the herbicide whose target is the HPPD can be applied in accordance with the invention, either before sowing the crop, before the crop emerges or after the crop emerges.

Within the meaning of the present invention, "herbicide" is understood as being a herbicidally active substance on its own or such a substance which is combined with an additive which alters its efficacy, such as, for example, an agent which increases its activity (a synergistic agent) or which limits its activity (a safener). The HPPD inhibitor herbicides are, in particular, as previously defined. It is of course to be understood that, for their application in practice, the above herbicides are combined, in a manner which is known per se, with the formulation adjuvants which are customarily employed in agricultural chemistry.

When the plant which has been transformed in accordance with the invention contains another gene for tolerance towards another herbicide (as, for example, a gene which encodes a mutated or unmutated EPSPS which confers on the plant tolerance to glyphosate), or when the transformed plant is naturally sensitive to another herbicide, the method according to the invention can comprise the simultaneous or chronologically staggered

application of an HPPD inhibitor in combination with the said herbicide, for example glyphosate.

The invention also relates to the use of the chimeric gene encoding a mutated HPPD as a marker gene during the "transformation/regeneration" cycle of a plant species and selection on the abovementioned herbicide.

The various aspects of the invention will be better understood with the aid of the experimental examples which follow.

All the methods or operations which are described below in these examples are given by way of example and correspond to a choice which is made from among the different methods which are available for arriving at the same result. This choice has no effect on the quality of the result and, as a consequence, any suitable method can be used by the skilled person to arrive at the same result. The majority of the methods for manipulating DNA fragments are described in "Current Protocols in Molecular Biology" Volumes 1 and 2, Ausubel F.M. et al., published by Greene Publishing Associates and Wiley Interscience (1989) or in Molecular cloning, T. Maniatis, E.F. Fritsch, J. Sambrook, 1982.

Example 1: Colorimetric test for screening for mutants which exhibit tolerance to 2-cyano-3-cyclopropyl-1-(2-SO_2CH_3-4-CF_3phenyl)propane-1,3-dione

pRP C: The vector pRP A (described in application WO 96/38567), which contains a genomic DNA fragment and the coding region of the gene for HPPD from *Pseudomonas fluorescens* A32, was digested with NcoI, purified and then ligated into the expression vector pKK233-2 (Clontech), which itself was digested with NcoI, the site for which forms a unique cloning site in this vector. The orientation of the gene in the resulting pRP C vector which permitted expression under the control of the *trc* promoter was verified.

A culture medium of the YT broth type, containing 1% agarose (Gibco BRL ultra pure), 5 mM L-tyrosine (Sigma) and the agent for selecting the abovementioned pRP C vector, is dispensed into a 96-well plate at the rate of 100 µl per well. 10 µl of a culture of E. coli in the exponential phase of growth and harbouring the pRP C vector are dispensed into each well. After 16 hours at 37°C, the wells which only contain the culture medium, or those which have been seeded with an E. coli culture harbouring the vector pKK233-2, are translucent whereas the wells which have been seeded with an E. coli culture harbouring the vector pRP C are coloured brown.

A series of samples was made up with identical culture medium which contained varying concentrations (0 mM to 14 mM) of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione (EP 0 496 631),

which was dissolved in water and brought to pH 7.5. This molecule is a diketonitrile which is recognized as being an effective inhibitor of HPPD activity (Pallett, K.E. et al., 1997. Pestic. Sci. 50, 83-84). The bacterial culture harbouring vector pRP C is observed to be totally without colour in the presence of a 7 mM concentration of the abovementioned compound.

HPPD mutants which were obtained by site-directed mutagenesis as well as by random mutagenesis were selected by rendering brown the medium containing the 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione at a concentration of 7 mM, as will be demonstrated below.

Identical results were obtained by substituting 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-(methylthio)phenyl)propane-1,3-dione and 2-(2-chloro-3-ethoxy-4-(ethylsulphonyl)benzoyl)-5-methyl-1,3-cyclohexadione (WO 93/03009) for 2-cyano-3-cyclopropyl-1-(2-methyl-4-trifluoromethylphenyl)-propane-1,3-dione. used at 3.5 mM and 7 mM, respectively.

These results confirm that a test which is based on HPPD activity, whatever the origin of this activity, makes it possible to identify HPPD activities which exhibit tolerance to HPPD activity inhibitors of the isoxazole family as well as of the triketone family.

Example 2: Random mutagenesis of the Pseudomonas fluorescens A32 HPPD gene using hydroxylamine.

The plasmid DNA of an E. coli culture harbouring the above-described pRP C vector was extracted using the standard protocol. This DNA was incubated, at 80°C for one hour, with hydroxylamine, which is a chemical mutagen which brings about the replacement of cytosine with thymidine, using a standard protocol. The E. coli K12 strain DH10B was transformed with the resulting potentially mutated plasmid DNA. Use of the colorimetric screening test described in Example 1 made it possible, after screening several thousand potentially mutated clones, to identify several colonies which were able to render the medium brown, that is to say able to transform HPP into homogentisate even in the presence of 7 mM 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione.

After sequencing these various mutants, it became evident that the use of this mutagenesis/screening method had resulted in the isolation of 4 distinct mutants corresponding to three distinct mutation sites:

- First site: proline 215 replaced by a leucine, which mutant was designated PfL215
- Second site:
  - glycine 334 replaced by a serine, which mutant was designated PfS334

- glycine 334 replaced by an aspartic acid, which mutant was designated PfD334

- Third site: glycine 336 replaced by a serine, which mutant was designated PfS336.

These three mutated sites, resulting in an improved tolerance being obtained compared with unmutated HPPD, are located in the C-terminal domain of the protein.

The clones derived from the clone pRP C which contain the coding region of the HPPD gene from Pseudomonas fluoroscens A32 containing one or more mutations are named pRP C followed by the name of the mutation(s) carried out, such as for example pRP C Pfl215 or pRPC PfS336 (or alternatively, simply by designating only the mutated amino acid, such as for example PfL215 or PfS336, unless otherwise indicated regarding the origin of the mutated HPPD).

The alteration(s) by means of random mutagenesis can be effected on any protein which has an HPPD-type activity, that is to say which transforms 4-hydroxyphenylpyruvate into homogentisate, and whose coding region is or could be cloned. While the HPPDs which are described in this text are, inter alia, those from P. fluorescens, Arabidopsis thaliana, Daucus carota, Zea mays and Synechocystis, it is certainly apparent to the skilled person that all these alterations can be applied to other HPPDs.

Example 3: Site-directed mutagenesis of the *Pseudomonas fluorescens* A32 HPPD gene by means of sequence analogy.

By means of aligning the protein sequences of the HPPDs from *Pseudomonas fluorescens* A32, *Arabidopsis thaliana*, mouse, pig and *Coccicoides*, it is possible to choose a certain number of the amino acids which are found to be conserved in different sequences and then to mutagenize them and obtain tolerant HPPDs; sequences of other HPPDs, described in the literature, could have been added to the alignment which is presented in Figure 1.

The alignment of these different sequences shows clearly that one of the best conserved regions is located between the glycine at position 332 and the phenylalanine at position 342. Not only is this region highly conserved but, in addition, it encompasses the two glycines in positions 334 and 336 which were identified by random mutagenesis (in bold in the sequence alignment and marked with a star). Mutagenesis was carried out on the pRP C vector using the Pharmacia U.S.E. mutagenesis kit. The M oligonucleotides were used for mutagenizing phenylalanine 333, glycine 334, glycine 336 and asparagine 337, and also for mutagenizing the double glycine 334 and glycine 336 mutants, as shown in the schemes below (appended sequence identifiers, SEQ ID NO.1 to 12):

Mutagenesis of PHE333, which is replaced exclusively by Trp

Oligo 1: GAAGTTGCCC TCGCCCCACC CATCGTCGCC CTT

Mutagenesis of PHE333, which is replaced exclusively by LEU & ILE

Oligo 2: GAAGTTGCCC TCGCCRAKCC CATCGTCGCC CTT

Mutagenesis of GLY334, which is replaced exclusively by TRP

Oligo 3: CTTGAAGTTG CCCTCCCAAA ACCCATCGTC GCC

Mutagenesis of GLY334, which is replaced exclusively by ASP

Oligo 4: CTTGAAGTTG CCCTCGTCAA ACCCATCGTC GCC

Mutagenesis of GLY334, which is replaced exclusively by SER

Oligo 5: CTTGAAGTTG CCCTCGCTAA ACCCATCGTC GCC

Mutagenesis of GLY334, which is replaced exclusively by LEU & ILE

Oligo 6: CTTGAAGTTG CCCTCRAKAA ACCCATCGTC GCC

Mutagenesis of GLY336 which is replaced exclusively by ASP

Oligo 7: CAGCGCCTTG AAGTTGTCCT CGCCAAACCC ATC

Mutagenesis of GLY336, which is replaced exclusively by GLU

Oligo 8: CAGCGCCTTG AAGTTYTCCT CGCCAAACCC ATC

Mutagenesis of GLY336, which is replaced exclusively by TRP

Oligo 9: CAGCGCCTTG AAGTTCCACT CGCCAAACCC ATC

Mutagenesis of GLY336, which is replaced exclusively by Ile

Oligo 10: CAGCGCCTTG AAGTTDACTC GCCAAACCCA TCMutagenesis of GLY334 & GLY336, which are replaced by all the other amino acids, and therefore with the possibility of obtaining a double mutant

Oligo 11: CGCTTGAAGT TNNNCTCNNN AAACCCATCG TC

Mutagenesis of ASN337, which is replaced exclusively by LEU & ILE

Oligo 12: GAACAGCGCC TTGAARAKGC CTCGCCAAA CCC

After screening several hundred potential mutants with 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylhenyl)propane-1,3-dione, 15 novel mutants, of which 12 were single mutants and 3 were double mutants (see summary table), were identified which exhibited tolerance to the 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione inhibitor in our colorimetric detection test. It is necessary to add that some of the mutants which were identified during the course of these site-directed mutageneses are certainly identical to the mutants which were identified in Example 2 (this is the case for PfS334 and PfD334).

It was possible to combine mutations, not only in close regions, as is the case for obtaining double mutants at positions 334 and 336, but also in distant regions, as is the case for obtaining double mutants at position 215 and 336, as indicated below.

pRP C PfL215 Delta : The plasmid DNA of the clone pRP C PfL215 was PstI digested, purified and then religated. Since the first PstI site is located at leucine 289, and the last PstI site is located in the multiple cloning site from PKK233.2, the clone obtained, pRPC PfL215 Delta, contains the portion of the coding sequence of HPPD PfL215 which includes the mutation Pro215Leu, but not the terminal portion or the genomic portion.

PRP C PfL215 W336, PRP C PfL215 E336, PRP C PfL215 I336 :

The plasmid DNAs of the clones pRP CPfW336, pRP CPfE336 and pRP CPfI336 were digested with MluI and HindIII, MluI being located in the coding portion of the HPPD from pseudomonas fluorescens, at aspartic acid 254, and HindIII being located in the multiple cloning site from PKK223.3. The fragments containing the C-terminal portions of the mutated HPPDs, as well as the genomic portion, were purified and then ligated into the plasmid pRP C PfL215 Delta, which had been digested with MluI and HindIII. The three clones thus obtained are equivalent to the clone pRP C PfL215, but also contain respectively the mutation W336, E336 and I336.

The mutants which were obtained are identified as follows:

Single mutants:

Phenylalanine333 replaced by tryptophan designated PfW333

Phenylalanine333 replaced by leucine	designated PfL333
Glycine334 replaced by tryptophan	designated PfW334
Glycine334 replaced by proline	designated PfP334
Glycine334 replaced by leucine	designated PfL334
Glycine334 replaced by isoleucine	designated PfI334
Glycine336 replaced by aspartic acid	designated PfD336
Glycine336 replaced by glutamine	designated PfQ336
Glycine336 replaced by glutamic acid	designated PfE336
Glycine336 replaced by tryptophan	designated PfW336
Glycine336 replaced by isoleucine	designated PfI336
Asparagine337 replaced by leucine	designated PfL337

Double mutants obtained by site-directed mutagenesis:

Glycine334 replaced by alanine and Glycine336 replaced by alanine	designated PfA334-A336
Glycine334 replaced by alanine and Glycine336 replaced by arginine	designated PfA334-R336
Glycine334 replaced by serine and Glycine336 replaced by arginine	designated PfS334-R336

Double mutants obtained by cloning:

Proline 215 replaced by leucine and Glycine 336 replaced by tryptophan	designated PfL215-W336
Proline 215 replaced by leucine and Glycine 336 replaced by glutamic acid	designated PfL215-E336

Proline 215 replaced by leucine and
Glycine 336 replaced by isoleucine designated PfL215-I336

This result demonstrates that it is possible, by means of mutagenizing the amino acids which are conserved between the protein sequences of different HPPDs and which are located in the C-terminal part of the protein, to obtain HPPDs which exhibit tolerance towards inhibitors of HPPD activity. Any region which is conserved between different HPPD amino acid sequences is therefore a good target for obtaining mutants which are advantageous to analyse in order to determine their tolerance. It is evident that any mutation or multiple mutation which would make it possible to obtain a tolerant HPPD, even if this protein is not exemplified in this text, is part of the subject-matter of the invention.

The result also demonstrates that the C-terminal domain is definitely the favoured target for mutagenizing an HPPD with a view to obtaining good tolerance of the enzyme towards these different inhibitors. Thus, it is very difficult to define a conserved region in the N-terminal domain, which is defined as proceeding from amino acid No. 1 to the linking peptide defined previously in the case of Pseudomonas fluorescens A32, or to its equivalents in the case of the HPPDs of other species.

The alteration(s) which is/are carried out by means of site-directed mutagenesis on the basis of

information deduced from a sequence alignment can be made to any protein which possesses an HPPD-type activity, that is to say which transforms 4-hydroxyphenylpyruvate into homogentisate and whose coding region is or could be cloned. Although the HPPDs which are described in this text are, inter alia, those from P. fluorescens, Arabidopsis thaliana, Daucus carota, Zea mays and Synechocystis, it will certainly be apparent that all these alterations can be applied to the other HPPDs.

Example 4: Site-directed mutagenesis of the Pseudomonas fluorescens A32 HPPD gene.

The results which were obtained by random mutagenesis as described in Example 2, as well as those obtained after aligning the different HPPD sequences, clearly demonstrate that the peptide proceeding from F#333 to F#338 (numbering on the P. fluorescens HPPD) is a region of particular interest in terms of mutagenizing in order to obtain tolerance.

In parallel with this information, it was found, by means of analysing the three-dimensional structure of the Pseudomonas fluorescens strain A32 enzyme, for example (Figure 1), that the C-terminal part of the protein monomer contains the catalytic site of the enzyme. It is therefore the amino acids of this C-terminal domain which have first and foremost to be mutagenized in the hope of being able to modify the

protein/inhibitor interaction and thereby obtain a more tolerant enzyme.

Point mutations which could lead to a conformational change of the Phe333 to Phe338 hexapeptide, without this peptide being directly mutated, have therefore been sought by analysing the three-dimensional structure of the P. fluorescens HPPD protein. The principal idea is that mutation of an amino acid which is rather distant from the active site and which is not conserved can influence the spatial positioning of this Phe333 to Phe338 peptide and therefore induce tolerance to HPPD inhibitors by means of an indirect effect.

Amino acids which are close to this hexapeptide were therefore sought in the structure; a small number of amino acids, such as the aspartic acid at position 287 and the glycine at position 298, meet this criterion [for information, the alpha carbon atom of glycine 334 of the hexapeptide is located 9.61 angstroms from the iron atom which is located in the catalytic site, and the alpha carbon of the glycine at position 298 is 5.75 angstroms from that of the glycine which is located at position 334]. Of the two amino acids, this glycine 298 appeared to be the best candidate, since any mutagenesis would lead to an amino acid which had a larger side chain, and this glycine was replaced by a glutamic acid (whose side chain is negatively charged) with a view to obtaining an

alteration which was sufficiently appreciable for the effect to be visible.

Furthermore, when the glycine at position 298 is replaced with a glutamic acid, the side chain of this glutamic acid, which side chain is very bulky, "knocks" against the beta-pleated sheet secondary structure in which the conserved LLQIF motif is located. The phenylalanine 312 of this motif is itself located very close to the iron.

The Gly298 to Glu298 mutation was tested.

This Gly298 to Glu298 mutation was brought about by site-directed mutagenesis (U.S.E. mutagenesis kit, Pharmacia) of the pRP C vector using the oligonucleotide No. 13 (appended sequence identifier - SEQ ID NO 13):

GLY298 replaced by glutamic acid

Oligo 13: GCCTTCCACGGAAGATTCGTCCAGCAGGATACC

This mutant, designated PfE298, causes the screening medium containing 7 mM RPA202248 to turn brown (see summary table).

This confirms that it is possible, knowing the three-dimensional structure of the HPPD, to identify a certain number of mutations which are effective in terms of tolerance towards inhibitors of the HPPD, whether the latter be of bacterial, plant or other origin. Thus, it is perfectly possible to model

the structure of any HPPD whose protein sequence is available, since the precise structure is known in the case of the P. fluorescens HPPD. This modelling will be of particular interest when modelling the C-terminal domain, which domain is that which is best conserved in terms of the primary sequence and, in particular, that which is most promising in terms of tolerance towards inhibitors of HPPD.

The alteration(s), which is/are brought about by site-directed mutagenesis on the basis of information deduced from the three-dimensional structure of a modelled HPPD, or the three-dimensional structure which is determined by analysing crystals obtained in the presence or absence of an inhibitor, can be effected on any protein which has an HPPD-type activity, that is to say which transforms 4-hydroxyphenylpyruvate into homogentisate and whose coding region is or can be cloned. While the HPPDs which are described in the present application are, inter alia, those of P. fluorescens, Arabidopsis thaliana, Daucus carota, Zea mays and Synechocystis, it will certainly be apparent to the skilled person that all these alterations can be applied to other HPPDs.

Example 5: Expression of mutant Pseudomonas fluorescens HPPDs in tobacco

A) Construction of chimeric genes

pRP-VB3 : The DNA of the binary vector pBI121 (Clontech) was digested with HindIII and XbaI and the ends were filled in with dNTPs using pfu polymerase (Stratagene); the vector was purified. The DNA of the clone pRP-S, which is described in Example 2 of the application PCT 96/38567 and which comprises the sequence "double histone promoter - TEV - OTP - HPPD gene - Nos terminator" was digested with HindIII and SacI and the ends were filled in with dNTPs using pfu polymerase (Stratagene); the purified insert was then ligated into the previously described purified vector.The orientation was verified by means of SalI digestion. The clone pRP-VB3 therefore has the following chimeric gene structure:

RB/Nos promoter/NPTII/Nos terminator/double histone promoter/tev/otp/HPPD/Nos terminator/LB

RB LB = right-hand and left-hand border respectively of the Agrobacterium tumefaciens T-DNA

Nos promoter = promoter of the Agrobacterium tumefaciens nopaline synthase

NPTII = coding sequence the type II neomycin phosphotransferase (giving resistance to kanamycin)

NOS terminator = terminator sequence of the Agrobacterium tumefaciens nopaline synthase

Double histone promoter = described in EP 507 689

tev = TEV enhancer (Carrington & Freed)

otp = optimized transit peptide (EP 508 909)

pRP-VB3-b : The DNA of clone pRP-VB3 was digested with BamHI, purified and then ligated into the vector pZERO-1 (Invitrogen), which does not contain the BstEII and SalI restriction sites and which was digested with BamHI, and the resulting vector was purified. A part of the OTP, the gene for HPPD and the Nos terminator are in this way transferred into pZERO-1.

pRP-VB3-c : The DNA of clone pRP-VB3-b was digested with SalI, purified and then ligated in the presence of the adapter shown below (oligonucleotides 14 and 15 - SEQ ID NO 14 and 15 appended) so as to replace the SalI restriction site with the BstEII restriction site.

5'TCGAGAGAGAGGTGACCGAGAGA 3'

3' CTCTCTCCACTGGCTCTCTAGCT 5'

pRP-VB3-d : The DNA of clone pRP-VB3-c was digested with BamHI, and the insert was purified and then cloned into the PUC19 vector (Biolabs) which had been digested with BamHI. A part of the OTP, the gene for HPPD and the Nos terminator are in this way transferred into PUC19.

pRP-VB3-e : Since the PUC19 vector does not possess PmlI and StuI restriction sites, the DNA of clone pRP-VB3-d was digested with PmlI and StuI, purified and then ligated to itself, thereby making it possible to delete the Not site in the HPPD gene and to

shorten the coding part of the HPPD by approximately 500 base pairs in order subsequently to facilitate screening of the transformed colonies which have integrated the mutant HPPDs.

pRP-VB3-f : Vector pRP-VB3-e was digested with NotI and the ends were filled in with dNTPs using pfu polymerase (Stratagene), after which the DNA was purified; it was then digested with BamHI, purified and then cloned into the KpnI-digested PUC19 vector, whose ends had been filled in with dNTPs using pfu polymerase (Stratagene), after which it was purified, digested with BamHI and purified.

pRP-VB83-g : The DNA of clone pRP-VB3 was digested with BstEII and the ends were filled in with dNTPs using pfu polymerase (Stratagene); the purified vector was then ligated to itself thereby making it possible to eliminate the unique BstEII site of this vector.

pRP-RD224 : The DNA of clone pRP-VB3-f was digested with BamHI and SacI, purified and then ligated into vector pRP-VB3-g, which had been digested with BamHI and SacI and purified. Clone pRP-RD224 therefore has the following structure:

RB/Nos promoter/NPTII/Nos terminator/double histone promoter/tev/otp/truncated HPPD/Nos terminator/LB

pRP-RD224 mutants : The DNAs of the vectors carrying the mutated HPPDs as well as the unmutated HPPD contained in vector PKK233-2 were digested with KpnI and BstEII, purified and then ligated into vector pRP-RD224, which had been digested with KpnI and BstEII and purified. The transformants which had integrated the mutated HPPD gene were selected for the size of the insert by digesting with KpnI and BstEII. The resulting clones are designated pRP-RD224 to which is added the type of mutation which has been carried out on the HPPD; in this way, the following clones were, for example, created: pRP RD224 Pf (for the unmutated enzyme), pRP RD224 PfD336 (for the enzyme having an aspartic acid at position 336), pRP RD224 PfQ336 (for the enzyme having a glutamine at position 336), pRP RD224 PfL333 (for the enzyme having a leucine at position 333) and pRP RD224 PfA334-A336 (for the enzyme having an alanine at position 334 and at position 336, i.e. a double mutant).

B) Transformation of "Petit havana" tobacco

The previously described chimeric genes were transferred into "Petit havana" tobacco using the transformation and regeneration procedures which have already been described in European application EP No. 0 508 909.

1) Transformation:

The vector is introduced into the non-oncogenic Agrobacterium tumefaciens strain EHA101.

2) Regeneration:

The "Petit havana" tobacco was regenerated from foliar explants on a basal Murashige and Skoog (MS) medium comprising 30 g/l sucrose as well as 350 mg/l cefotaxime and varying doses, i.e. 10 ppm, 20 ppm and 40 ppm, of 2-cyano-1-[4-(methylsulphonyl)-2-trifluoromethylphenyl]-3-(1-methylcyclopropyl)propane-1,3-dione (EP 496630), which is an analogue of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione, or directly doses of 2 or 4 or 8 ppm of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione. The foliar explants are removed from greenhouse plants and transformed by the foliar disc technique (Science 1985, Vol. 227, p. 1229-1231) in three consecutive stages:

- the first comprises inducing shoots on an MS medium to which is added 30 g/l sucrose and which contains 0.05 mg/l naphthylacetic acid (ANA) and 2 mg/l benzylaminopurine (BAP) for 15 days and in the presence of varying doses of herbicide, i.e. 2-cyano-1-[4-(methylsulphonyl)-2-trifluoromethylphenyl]-3-(1-methylcyclopropyl)propane-1,3-dione (EP 496630) or 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione.

- The green shoots which are formed during this stage are then developed by culturing them, for 10 days, on an MS medium to which is added 30 g/l

sucrose and which contains varying doses of herbicide but does not contain any hormone.

- Developed shoots are then removed and cultured on an MS rooting medium containing half concentrations of salts, vitamins and sugars and varying doses of isoxaflutole but not containing any hormone. After about 15 days, the rooted shoots are placed in soil.

C) Measuring the tolerance of the plantlets to herbicide in vitro.

The experiments are carried out by reacting a selective agent with the mutants indicated in the following table. They demonstrate that while shoots/plantlets are not obtained at the highest doses of herbicide in the transformation/regeneration assays using an unmutated HPPD, the mutants, by contrast, make it possible, due to the tolerance of the enzyme being improved, to obtain plantlets even at high doses of 2-cyano-1-[4-(methylsulphonyl)-2-trifluoro-methylphenyl]-3-(1-methylcyclopropyl)propane-1,3-dione (EP 496630) or of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione (see summarizing table). These plantlets, which are obtained at high concentrations of the selective agent when a mutated HPPD is used and which it is not possible to obtain when wild-type HPPD is used, are completely normal.

In the in vitro selection on 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propan-1,3-dione at 1 ppm, 2 ppm, 4 ppm and 8 ppm, it appears that a certain number of mutants do not allow the easy regeneration of transformed tobacco plantlets, whatever the dose of herbicide used. It will be noted, however, that transformants may be obtained using a high number of transformation attempts, and/or by determining the concentrations of selection agent.

In contrast to other mutants, it is easy to obtain the plantlets which are green and thus completely normal. The mutants which give most plantlets are the mutants PfL215, PfD336 and PfQ336 (for the mutants studied according to this method).

In the transformation with PfD336,
25% of the transformation events tolerating the selection made at 1 ppm was obtained,
25% of the transformation events tolerating the selection made at 2 ppm was obtained,
35% of the transformation events tolerating the selection made at 4 ppm was obtained,
15% of the transformation events tolerating the selection made at 8 ppm was obtained.

In the transformation with PfL215,
8% of the transformation events tolerating the selection made at 1 ppm was obtained,

60% of the transformation events tolerating the selection made at 2 ppm was obtained,
8% of the transformation events tolerating the selection made at 4 ppm was obtained,
24% of the transformation events tolerating the selection made at 8 ppm was obtained.

In the transformation with PfQ336,
35% of the transformation events tolerating the selection made at 1 ppm was obtained,
25% of the transformation events tolerating the selection made at 2 ppm was obtained,
40% of the transformation events tolerating the selection made at 4 ppm was obtained,
0% of the transformation events tolerating the selection made at 8 ppm was obtained.

In the transformation with the unmutated HPPD,
15% of the transformation events tolerating the selection made at 1 ppm was obtained,
70% of the transformation events tolerating the selection made at 2 ppm was obtained,
15% of the transformation events tolerating the selection made at 4 ppm was obtained,
0% of the transformation events tolerating the selection made at 8 ppm was obtained.

These results thus confirm that the mutated HPPDs are statistically more effective than the wild-type HPPD; with the two mutants PfD336 and PfL215 it is

possible to obtain transformants at the highest dose of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propan-1,3-dione, whereas it is not possible with the unmutated HPPD.

In addition, with the mutant PfQ336, many transformation events are obtained at 4 ppm of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propan-1,3-dione, 40% of the plantlets, whereas with the wild-type HPPD a low percentage of the plants are obtained at 4 ppm of this herbicide.

Table summarizing the mutants of P. fluorescens HPPD

HPPD type	Activity in the screening test using a selective agent dose of		% of green plantlets obtained at a dose of x ppm of herbicide
	0 mM	7 mM	
Wild type	10	0	15% at 4 ppm
Mutated			
PfL215	10	7	24% at 8 ppm
PfE298	8	3	no green plantlets
PfL333	5	4	-
PfW333	-	-	no green plantlets
PfS334	7	7	no green plantlets
PfD334	1	1	-

PfW334	6	6	-
PfP334	5	5	no green plantlets
PfL334	5	4	no green plantlets
PfI334	5	4	no green plantlets
PfS336	-	-	-
PfD336	2	1	15% at 8 ppm
PfQ336	8	8	40% at 8 ppm
PfE336	10	8	-
PfW336	10	9	-
PfI336	10	8	-
PfL337	8	8	no green plantlets
PfA334-A336	8	8	-
PfA334-R336	8	7	no green plantlets
PfS334-H336	5	5	no green plantlets

*: screening test described in Example 1.

'-' data not available.

The figure 10 corresponds to a high activity of the same level as that obtained with unmutated HPPD in the absence of inhibitor, while the figure 0 corresponds to no activity, either due to complete inhibition by 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione or due to a mutation which results in the enzyme becoming inactive; the figures 1 to 9 correspond to intermediate activities, with the activity increasing as the figure increases in the evaluation test described in Example 1.

These results confirm that introducing an HPPD exhibiting a tolerance to inhibitors of HPPD activity into plants confers a tolerance to these same inhibitors on the plants, which tolerance is therefore superior to that which is obtained with an unmutated HPPD.

D) Tolerance *in vivo*.

All the plants, after rooting, were acclimatized to the greenhouse and taken through to seed.

The seeds of several capsules are mixed, and then sown on a mixture of compost and sand, in a proportion of 100 seeds approximately per seedling tray. The Petit Havana wild-type genotype is included as a control. The treatments are carried out in a proportion of 600 g/ha of formulated isoxaflutole. Seedling appearance is graded 12 days after treatment, the grade 5 being for the best appearance, the grade 1 being for the worst. The means of the observations of several transformation events (10-20 transformation events per HPPD evaluated) are representative of the values of pre-emergence tolerance of the wild-type HPPD and of the mutated HPPDs.

These measurements carried out for several *Pseudomonas fluorescens* HPPD mutants on positions 336 and 215 make it possible to establish the mean values of tolerance in the table below.

Test	1	2
Unmutated Pf	2.06	2.2
PfW336	-	2.8
PfD336	3.35	-
PfQ336	2.94	-
PfL215	3	-

These results show that the mutated HPPDs according to the invention have, in the transformed plants, a tolerance to HPPD-inhibiting herbicides which is greater than the tolerance obtained with the corresponding native HPPD.

Example 6: Site-directed mutagenesis of the *Synechocystis* HPPD gene.

A mutation site which is effective in terms of tolerance to HPPD inhibitors is chosen in the *Pseudomonas fluorescens* HPPD and the site is transposed to another HPPD.

This transposition is made to an HPPD which is as different as possible from the HPPD of *Pseudomonas fluorescens* and from other known HPPDs. The gene which was chosen for this work was the gene encoding *Synechocystis* HPPD, which gene was known due to the systematic sequencing of the genome of this cyanobacterium but had never been cloned as such. The gene was therefore first of all isolated and then expressed in *E. coli*.

1) Isolation of the gene.

The genomic DNA of the cyanobacterium *Synechocystis* PCC6803 was extracted and purified using the standard protocols. 200 µg of this genomic DNA were amplified by polymerization chain reaction (PCR) using 1.25 U of pow polymerase (Boehringer) in its buffer, in a reaction volume of 50 µl containing 200 µM of dNTP (deoxyribonucleotide triphosphate). The synthetic oligonucleotide sequences 16 and 17 (SEQ ID NO 16 and 17 appended), which were used as primers were deduced from the sequence of *Synechocystis* HPPD which was published in Genebank.

Oligo 16 ATTATGGAAT TCGACTATCT T

Oligo 17 CAGTATTCAT AATGTTAATT ATG

The amplification programme, i.e. 5 minutes at 94°C, then 50 cycles of 1 minute at 94°C, 1 minute at 49°C and 1 minute at 72°C, then 5 minutes at 72°C, was carried out using a Perkin-Elmer 9600 apparatus.

2) Cloning and expression of the gene.

The amplified fragment which was obtained by PCR was purified, digested with EcoRI, repurified and then cloned into the vector ptrc-99A (Pharmacia), which had previously been digested with EcoRI and SmaI. The bacterium JM105 was transformed with the recombinant plasmid. The conformity of the cloned fragment with the published *Synechocystis* HPPD sequence was verified by sequencing.

The dioxygenase activity of the *Synechocystis* HPPD which had thus been obtained was observed by the browning of the medium, using the previously described colorimetric test (cf. Example 1), with addition of IPTG (isopropyl-β-D-thiogalactopyranoside) at a concentration of 1 mM in order to induce expression of the protein. Under the same conditions, but in a medium containing 7 mM 2-cyano-3-cyclopropyl-1-(2-methyl-sulphonyl-4-trifluoromethylphenyl)propane-1,3-dione, there is no browning of the medium, thereby confirming inhibition of the *Synechocystis* HPPD activity by the 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione.

3) Site-directed mutagenesis.

By means of aligning the protein sequences of the *Pseudomonas fluorescens* A32 HPPD and of the *Synechocystis* PCC6803 HPPD, it was estimated that the glycines in positions 334 and 336 of the *Pseudomonas* HPPD (glycines which are indicated by stars in the figure of Example 3 and which are very highly conserved) are in positions 318 and 320 in the *Synechocystis* HPPD (in bold in the protein sequence alignment in Figure 3.

With several mutants at position 334 in *Pseudomonas fluorescens* having been obtained, two site-directed mutagenesis experiments (U.S.E., Pharmacia) were carried out using the oligonucleotides MUGLYA and MUGLYB, which oligonucleotides were intended to replace

the glycine at position 318 (of *Synechocystis* corresponding to the glycine at position 334 of *P. fluorescens*) either with an asparagine or a serine or with a proline or an alanine (SEQ ID NO 18 and 19).

GLY318, possible replacement with SER & ASN

Oligo 18 CGGGCAAAAG GATTTARCCA AGGAAACTTT CAAG

GLY318, possible replacement with PRO & ALA

Oligo 19 CGGGCAAAAG GATTTSCNCA AGGAAACTTT CAAG

In the two experiments, clones obtained after mutagenesis caused browning of the screening medium at 7 mM 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione (test described in Example 1). One mutant from each experiment was sequenced.

Glycine318, replaced with asparagine, designated SyN318, similar to PfD334 (in Example 3).

Glycine318, replaced with alanine, designated SyA318, similar to PfA334 (in Example 3).

These results confirm that mutations leading to tolerance, which tolerance is demonstrated for a given HPPD, are transposable to another HPPD which belongs to another species and another kingdom.

These results also confirm that alterations of the protein sequence in the C-terminal part of an HPPD can, whatever the origin of the HPPD (bacterial or other origin) result in tolerance towards HPPD inhibitors.

Example 7: Biochemical study of a mutated HPPD; mutants of *Synechocystis*.

The mutants SyN318 and SyA318 which were obtained in the previous example were examined, in comparison with the unmutated HPPD of *Synechocystis*, for the biochemical characteristics K_m and IC_{50} with regard to an HPPD inhibitor, i.e. 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)-propane-1,3-dione. This analysis was carried out using the following protocol:

a) Measuring activity

The HPPD activity is measured by determining, by means of high performance liquid chromatography (HPLC), the quantity of product, i.e. homogentisate, which is formed after incubating the enzyme with its substrate HPP. Injecting different amounts of homogentisate, varying from 12 to 48 nmoles, into the column enables the retention time of the homogentisate to be determined and the peak area to be correlated with the quantity of product injected.

The activity measurements are carried out in a final volume of 200 µl containing: 12.6 mM ascorbate, 178 µM iron ($FeH_8N_2O_8S_2, 6H_2O$) (previously prepared in 0.1 M tris-acetate buffer, pH 6), 50 µg of crude extract containing the HPPD, 352 µM HPP and 0.1 M tris-acetate buffer, pH 7.5. The enzyme is firstly incubated with the iron at 30°C for 1 min and then with the ascorbate at 30°C for 5 min before the reaction is

started by adding the substrate, i.e. the HPP. The incubation is continued at 30°C for 1 min 30 sec and the reaction is then stopped by adding 70 µl of 20% perchloric acid. The proteins are then removed by centrifuging for 5 min at 15,300 rpm and at 20°C. The supernatant is recovered. The quantity of homogentisate formed is then analysed by injecting 75 µl of assay mixture into a Pico Tag C18 column which is connected to the HPLC system. Elution is carried out at a flow rate of 1 ml/min. The isocratic elution which is carried out is as follows: 1- 0% of buffer B (that is 100% of buffer A: water, 3% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid) for 6 minutes; 2- 100% of buffer B (100% acetonitrile) up to the 11th minute; 3- 0% of buffer B up to the 19th minute. On leaving the column, the homogentisate is detected by measuring absorbance at 288 nm. The quantity of product formed is defined by the area of the peak on the chromatogram.

b) Determination of the K_m

The K_m of the HPPD for HPP is determined by measuring the initial velocity of the reaction using different concentrations of HPP. The reactions are carried out under the above-described conditions using HPP concentrations of from 5.5 µM to 1400 µM.

c) Determination of the IC_{50}

The IC_{50} is determined by measuring the initial velocity of the reaction under the above-described conditions after incubating the enzyme

at 30°C for 10 min with the iron, the ascorbate, the substrate at a concentration of 1056 µM and varying concentrations of inhibitor. The concentrations of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione employed vary from 10^{-10} to 10^{-4} M.

The K_m values of the enzyme for its substrate, i.e. 4-hydroxyphenylpyruvate, which were calculated, and the IC_{50} values which were calculated at comparable activities, are recorded in the table below:

	Native HPPD	SyN318	SyA318
K_m	60 µM	470 µM	320 µM
IC_{50}	80 nM	80 µM	40 µM

These results confirm that while the mutations which are carried out in the C-terminal part of the protein exert an influence by diminishing the affinity of the enzyme for the substrate (K_m), they exert an even stronger influence by diminishing the affinity of the enzyme for the inhibitor (IC_{50}). Thus, the ratio of the IC_{50} of a mutant HPPD to the IC_{50} of the non-mutant HPPD (which reflects the loss of affinity for the inhibitor) is 1000 and 500 for SyN 318 and SyA 318, respectively, while the ratio of the K_m of a mutant HPPD to the K_m of the non-mutant HPPD (which reflects the loss of affinity for the substrate) is 8 and 5 for SyN 318 and SyA 318, respectively. This illustrates

very well the fact that, while these two mutants have a slightly lower affinity for the substrate of the enzyme, they in particular have a markedly lower affinity for inhibitors of the enzyme, including 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione.

These results confirm that the colorimetric screening test described in Example 1 does indeed make it possible to detect mutated HPPDs which exhibit tolerance to inhibitors of HPPD activity. They validate the method which is being used to screen and identify HPPD mutants which are tolerant towards inhibitors of HPPD. While this screening method is rapid and simple to use, and this is why it is being employed, it is clear that any other method can be used in order to make a similar analysis; it would be possible to use

- a screening method which measures activity, and its inhibition, and which is based on the disappearance of HPP (radioactive assay, spectrophotometric assay or other assay) or based on the consumption of oxygen or based on the appearance of homogentisate (with coupling to another enzyme activity)

- an in vivo screening method, such as the growth of bacteria on HPP as the sole carbon source in the presence of inhibitors of HPPD, thereby making it possible to select the only clones having a tolerant HPPD

- it is similarly entirely possible to envisage using an in vivo screening in plants by employing plant transformation vectors, transformation systems, regeneration systems and selection systems with an inhibitor of HPPD, such as those described in Examples 5 and 8.

Example 8: Evaluation of the unmutated and mutated HPPD of Synechocystis; SyN318 and mutated SyA318 in tobacco.

A) Construction of the chimeric genes:

The vector which is employed in order to make the construct which enables *Synechocystis* HPPD (wild-type or mutant) to be expressed in type PBD6 tobacco plants is designated pRD224 (described in Example 5). This vector was initially conceived for cloning all the *Pseudomonas* HPPD mutants by simply replacing the truncated HPPD gene of this vector between the KpnI and BstEII sites. It can also be used to clone the *Synechocystis* HPPD gene with a view to creating a transgenic plant.

B) Cloning strategy

The sequence encoding *Synechocystis* HPPD is cloned into the pRD224 vector by replacing the sequence encoding the truncated *Pseudomonas fluorescens* HPPD. However, the *Synechocystis* HPPD sequence cannot be cloned directly between the KpnI and BstEII sites since the N-terminal sequences of the *Synechocystis* and *Pseudomonas* HPPD genes are very different. However, it is possible to clone between the BamHI site of the OTP

and the BstEII site. In order to do this, it is necessary to recreate, from the 5' end, upstream of the HPPD sequence, the BamHI site followed by the part encoding OTP which is located downstream of BamHI.

The *Synechocystis* HPPD gene which is present in the vector pTRC 99A is amplified by polymerization chain reaction (PCR) using primers A and B. The oligonucleotide A makes it possible to add the BamHI site upstream of the HPPD gene as well as a part of the OTP sequence between the BamHI site and the beginning of the gene. The oligonucleotide B makes it possible to add the BstEII site downstream of the gene. Primers A and B are depicted in SEQ ID NO 20 and 21:

oligonucleotide A:

5'NNNNNNNNNN ***GGATCC***GGTG CATGGAATTC GACTATCTTC3'

oligonucleotide B:

5'NNNNNNNNNN ***GGTCACC***AGT ATTCATAATG TTAATTATG3'

The amplification reaction is carried out at a hybridization temperature of 52°C. The PCR products are then separated on an agarose gel. The DNA band corresponding to the HPPD gene is cut out and purified.

The fragments which have thus been amplified are digested with BamHI at 37°C for 1 hour and then with BstEII at 60°C for 1 hour. This insert is then isolated on an agarose gel. The DNA band corresponding to the HPPD gene is cut out and purified.

This fragment is then cloned into the binary vector. The latter has previously been digested with

BamHI and BstEII and then separated from the fragment corresponding to the truncated HPPD on an agarose gel. This vector is then purified in the same manner as the HPPD gene.

The ligation between the binary vector and the insert is carried out at 16°C overnight using T4 DNA ligase. The ligation mixture is used to transform electrocompetent *E. coli* DH10B cells. The positive clones are selected on LB medium containing 50 µg/ml kanamycin. The presence of the insert of interest in the binary vector is checked on an agarose gel after carrying out a minipreparation and digesting the plasmid DNA with BamHI and SacI at 37°C for 1 hour. The recombinant vector is then used to transform electrocompetent Agrobacterium tumefaciens EHA105 cells. The selection is carried out on LB medium containing 50 µg/ml kanamycin. This recombinant vector is therefore carrying a T-DNA containing the gene for resistance to kanamycin, and a sequence encoding an OTP-Synechocystis HPPD unit under the control of the double histone promoter and a Nos terminator.

C) Transformation/regeneration of the PBD6 tobacco variety

The transformation is performed as described in Example 5 except that the selection is first of all carried out using 200 mg/ml kanamycin.

On the other hand, the young shoots which are obtained on kanamycin are excised and transferred

individually onto a medium lacking hormones, in order to promote their rooting, and containing 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethyl-phenyl)propane-1,3-dione in order to select the transgenic plantlets which are tolerant to this herbicide. The medium is MS medium (SIGMA M-5519 4.4 g/l) containing 350 mg/l cefotaxime, 1% sucrose (w/v) and 0 or 8 ppm of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione. The overproduction of HPPD in the transformed cells enables chlorophyllous plantlets to develop which are tolerant to 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione, while the plantlets which are derived from untransformed cells, and which are sensitive to the herbicide, appear totally white.

After two weeks, the roots are sufficiently developed for the plantlets to be transferred into soil and cultivated in a greenhouse.

D) Results

In the case of each construct, approximately 40 shoots are regenerated from an average of 60 foliar discs. After 2 days of culture on a medium in the presence of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione, some plantlets begin to turn white. After 8 days of rooting, this whitening is sufficiently significant to be interpretable. At 8 ppm of 2-cyano-3-cyclopropyl-

1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione, it is observed that only 40% of the plants harbouring the wild-type enzyme survive as against 72% for the plants harbouring the enzyme SyA318 and 88% for those harbouring the enzyme SyN318. The plants harbouring the mutated enzymes therefore exhibit a tolerance which is superior to that of the plants harbouring the wild-type enzyme.

After more than a month on 8 ppm of 2-cyano-3-cyclopropyl-1-(2-methylsulphonyl-4-trifluoromethylphenyl)propane-1,3-dione, the number of green plantlets in the case of transformation with the wild-type HPPD is 0%, while in the case of SyN318 this percentage is 17% and in the case of the mutant SyA318 it is 19%.

In parallel, if the regeneration is carried out in the presence of concentrations of from 5 to 10 ppm of 2-cyano-1-[4-(methylsulphonyl)-2-trifluoromethylphenyl]-3-(1-methylcyclopropyl)propane-1,3-dione (EP 496630) (a weaker inhibitor of the HPPDs), the three genes make it possible to obtain plants which are morphologically entirely in keeping. This confirms, if this were necessary, that overexpression of a mutated or unmutated HPPD makes it possible to obtain tolerance in the laboratory.

These results appear to be in agreement with the results obtained *in vitro* by means of enzyme kinetics and it appears to be definitely possible to establish a correlation between the *in vitro*

biochemical measurements and the results obtained *in vivo*.

This latter example confirms that there is at least partial consistency between the screening in vitro (Example 1), the biochemical analysis (Example 6) and the tolerance of a plant.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

EDITORIAL NOTE FOR

11603/99

THE FOLLOWING SEQUENCE LISTING IS PART OF THE DESCRIPTION

THE CLAIMS FOLLOW ON PAGE 63

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: RHONE-POULENC AGROCHIMIE

(B) STREET: 14-20 Rue Pierre BAIZET

(C) CITY: LYON

(E) COUNTRY: France

(F) POSTAL CODE: 69009

(ii) TITLE OF THE INVENTION: Mutated hydroxyphenylpyruvate dioxygenase, DNA sequence and isolation of plants which contain such a gene and which are tolerant to herbicides

(iii) NUMBER OF SEQUENCES: 21

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAAGTTGCCC TCGCCCCACC CATCGTCGCC CTT 33

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAAGTTGCCC TCGCCRAKCC CATCGTCGCC CTT 33

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTTGAAGTTG CCCTCCCAAA ACCCATCGTC GCC 33

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTTGAAGTTG CCCTCGTCAA ACCCATCGTC GCC 33

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTTGAAGTTG CCCTCGCTAA ACCCATCGTC GCC 33

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTTGAAGTTG CCCTCRAKAA ACCCATCGTC GCC 33

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CAGCGCCTTG AAGTTGTCCT CGCCAAACCC ATC 33

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CAGCGCCTTG AAGTTYTCCT CGCCAAACCC ATC 33

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CAGCGCCTTG AAGTTCCACT CGCCAAACCC ATC 33

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAGCGCCTTG AAGTTDACTC GCCAAACCCA TC 32

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CGCTTGAAGT TNNNCTCNNN AAACCCATCG TC 32

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GAACAGCGCC TTGAARAKGC CCTCGCCAAA CCC 33

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GCCTTCCACG GAAGATTCGT CCAGCAGGAT ACC 33

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TCGAGAGAGA GGTGACCGAG AGA 23

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TCGATCTCTC GGTCACCTCT CTC 23

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ATTATGGAAT TCGACTATCT T 21

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CAGTATTCAT AATGTTAATT ATG 23

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CGGGCAAAAG GATTTARCCA AGGAAACTTT CAAG 34

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CGGGCAAAAG GATTTSCNCA AGGAAACTTT CAAG 34

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

NNNNNNNNNN GGATCCGGTG CATGGAATTC GACTATCTTC 40

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

NNNNNNNNNN GGTCACCAGT ATTCATAATG TTAATTATG 39

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated functional mutated HPPD which is less sensitive to HPPD inhibitors, characterized in that it contains at least one mutation in its C-terminal part.

2. Mutated HPPD according to claim 1, characterized in that the C-terminal part of the enzyme differs from its N-terminal part by a linking peptide.

3. Mutated HPPD according to claim 2, characterized in that the linking peptide representing the N-terminal end of the C-terminal part of the HPPD is located between 5 and 15 amino acids upstream of the amino acid Asp161, with reference to the *Pseudomonas* HPPD.

4. Mutated HPPD according to one of claims 1 to 3, characterized in that the C-terminal part consists of the protein sequence which is contained between the linking peptide, and the C-terminal end of the enzyme.

5. Mutated HPPD according to one of claims 1 to 4, characterized in that the mutation is effected on amino acids which are replaced with amino acids which exhibit greater steric hindrance.

6. Mutated HPPD according to one of claims 1 to 5, characterized in that the mutation is effected on amino acids which exhibit low steric hindrance.

7. Mutated HPPD according to claim 6, characterized in that the amino acid of low steric hindrance is glycine (Gly).

8. Mutated HPPD according to one of claims 1 to 7, characterized in that the amino acid of the mutation site is replaced with one of the following amino acids: glutamine (Gln), glutamic acid (Glu), leucine (Leu), isoleucine (Ile) or tryptophan (Trp).

9. Mutated HPPD according to one of claims 1 to 8, characterized in that the mutation is effected on an amino acid of the C-terminal part which is common to several HPPD sequences.

10. Mutated HPPD according to one of claims 1 to 9, characterized in that it contains, in its C-terminal part, the following peptide sequence:
- Gly - Phe - Xaa - Yaa - Xab - Asn - Phe - Yab - Yac - Leu - Phe -

in which Xaa and Xab, independently of each other, represent glycine (Gly) or an amino acid which exhibits a hindrance which is greater than that of glycine, with it being understood that if either Xaa or Xab represents Gly, the other amino acid is then different from Gly,

Yaa represents any amino acid, preferably Ala, Lys or Glu,

Yab represents any amino acid, preferably Lys, Ser, Arg or Asn, and

Yac represents any amino acid, preferably Ala, Ser, Glu or Gln.

11. Mutated HPPD according to claim 10, characterized in that at least one of Xaa and Xab represents Leu, Glu, Trp or Ile.

12. Mutated HPPD according to claim 11, characterized in that Xab represents Glu, Trp or Ile, preferably Trp.

13. Mutated HPPD according to one of claims 1 to 12, characterized in that, with reference to the *Pseudomonas* HPPD sequence, the mutated amino acids are selected from the following amino acids: Pro215, Gly298, Gly332, Phe333, Gly334, Gly336 and Asn 337, preferably the amino acids Pro215 and Gly336.

14. Mutated HPPD according to claim 13, characterized in that it contains a mutation which is selected from the following mutations: Pro215Leu, Gly336Glu, Gly336Trp or Gly336Ile.

15. An isolated nucleic acid sequence which encodes a mutated HPPD according to one of claims 1 to 14.

16. Chimeric gene which comprises a coding sequence as well as heterologous regulatory elements, in the 5' and 3' positions, which are able to function in a host organism, in particular plant cells or plants, characterized in that the coding sequence contains at least one nucleic acid sequence which encodes a mutated HPPD as defined in any one of claims 1 to 14.

17. Chimeric gene according to claim 16, characterized in that the host organism is selected from bacteria, for example *E. coli*, yeasts, in particular the genera *Saccharomyces* or *Kluyveromyces*, *Pichia*, fungi, in particular *Aspergillus*, baculoviruses or plant cells and plants.

18. Chimeric gene according to claim 17, characterized in that the host organism is a plant cell or a plant.

19. Chimeric gene according to claim 18, characterized in that it contains, 5' of the nucleic acid sequence which encodes a mutated HPPD, a nucleic acid sequence which encodes a plant transit peptide, with this sequence being arranged between the promoter region and the sequence encoding the mutated HPPD so as to permit expression of a transit peptide/mutated HPPD fusion protein.

20. Transit peptide/mutated HPPD fusion protein, with the mutated HPPD being defined in accordance with one of claims 1 to 14.

21. Cloning and/or expression vector for transforming a host organism, characterized in that it contains at least one chimeric gene according to one of claims 16 to 19.

22. Method for transforming a host organism, characterized in that at least one nucleic acid sequence according to claim 15 or one chimeric gene

according to one of claims 16 to 19 is stably integrated into the said host organism.

23. Method according to claim 22, characterized in that the host organism is a plant cell.

24. Method according to claim 23, characterized in that a plant is regenerated from the transformed plant cell.

25. Transformed host organism, in particular a plant cell or plant, characterized in that it contains a nucleic acid sequence according to claim 15 or a chimeric gene according to one of claims 16 to 19.

26. Plant cell, characterized in that it contains a nucleic acid sequence according to claim 15 or a chimeric gene according to one of claim 16 to 19.

27. Transformed plant, characterized in that it contains transformed cells according to claim 26.

28. Plant according to claim 27, characterized in that it is regenerated from the transformed cells according to claim 26.

29. Transformed plant, characterized in that it is derived by cultivating and/or crossing the regenerated plants according to claim 28.

30. Transformed plants according to one of claims 27 to 29, characterized in that they are selected from monocotyledones, in particular cereals, sugar cane, rice and maize, or dicotyledones, in

particular tobacco, soya bean, rape, cotton, beetroot and clover.

31. Seeds of the transformed plants according to one of claims 27 to 30.

32. Method for controlling weeds in an area of a field which contains transformed seeds according to claim 31 or plants according to one of claims 27 to 30, which method consists in applying, to the said area of the field, a dose of an HPPD inhibitor herbicide which is toxic for the said weeds, without, however, significantly affecting the seeds or plants which have been transformed with the said chimeric gene according to the invention.

33. Method for cultivating the plants which have been transformed according to one of claims 27 to 30, which method consists in planting the seeds of the said transformed plants according to claim 31 in an area of a field which is appropriate for cultivating the said plants, in applying, if weeds are present, a dose, which is toxic for the weeds, of a herbicide whose target is the HPPD to the said area of the said field, without significantly affecting the said transformed seeds or the said transformed plants, and in then harvesting the cultivated plants when they reach the desired stage of maturity and, where appropriate, in separating off the seeds of the harvested plants.

34. Method according to either of claims 32 and 33, characterized in that the HPPD inhibitor is selected from isoxazoles, in particular isoxaflutole, diketonitriles, in particular 2-cyano-3-cyclopropyl-1-(2-SO_2CH_3-4-CF_3phenyl)propane-1,3-dione and 2-cyano-3-cyclopropyl-1-(2-SO_2CH_3-4-2,3 Cl_2 phenyl)propane-1,3-dione, triketones, in particular sulcotrione, or pyrazolinates.

```
Mus musculus                    ------NTTYNN--KGPKPERG----------------------RPLHFHS
Coccidioides immitis            MAPAADSPTLQ-----PAQPSD------------LN------QYRGYDH
Mycosphaerella graminicola      MAPGALLVTSQNGRTSPLYDSDGYVP---APAALVVGGE---VNYRGYHH
Hordeum vulgare                 MPPTPTTPAA-TGAAAAVTPEHARP----HR-MVRFNPRSDRFHTLSFHH
Zea mais                        MPPTPTAAAAGAAVAAASAAEQAAFRLVGHRNFVRFNPRSDRFHTLAPHH
Arabidopsis thaliana            MGHQNAAVSE-NQNHDDQAASSPQFKLVGFSKFVRKNPKSDKFKVKRPHH
Daucus carota                   MGKK-QSEAE-ILSSNSSNTSPATFKLVGFNNFVRANPKSDHFAVKRPHH
Streptomyces avermitilis        ----MTQTTEHT----PDTARQADP----------------FPVKGMDA
Pseudomonas fluorescens         -----MADLYEN-------PMG----------------------LMGPEP

P. fluorescens numbering        !                 !
                                1                 10

Mus musculus                    VTFHVGNAKQAASFYCNKMGFEPLAYRGLETGSREVVSHVIKRGKIVFVL
Coccidioides immitis            VEWYVGNAKQAATYYVTRMGFERVAYRGLETGSEAVASHVVRNGNITFIL
Mycosphaerella graminicola      AEWHVGNAKQVAQFYITRMGFEPVAHKGLETGSRFFASHVVQNNGVRFVF
Hordeum vulgare                 VEFWCADAASAAGRFAFALGAPLAARSDLSTGNSAHASQLLRSGSLAFLF
Zea mais                        VELWCADAASAAGRFSFGLGAPLAARSDLSTGNSAHASLLLRSGSLSFLF
Arabidopsis thaliana            IEFWCGDATNVARRFSWGLGMRPSAKSDLSTGNNVHASYLLTSGDLRFLF
Daucus carota                   IEFWCGDATNTSRRFSWGLGMPLVAKSDLSTGNSVHASYLVRSANLSFVF
Streptomyces avermitilis        VVFAVGNAKQAA-EYSTAFGMQLVAYSGPENGSRETASYVLTNGSARFVL
Pseudomonas fluorescens         IKLASPTPNTLEPIFEEMGPTKVATHR-----SKD--VHLYRQGAINLIL
                                                .  :  :   .   :      :::

                                !         !         !         !
                                20        30        40        50

Mus musculus                    CSALN----------PWN--------KRMGDHLVKHGDGVKDIAFEVKDC
Coccidioides immitis            TSPLR-SVEQA---SRFP--EDEALLKEIHAHLERHGDGVKDVAFEVDCV
Mycosphaerella graminicola      TSPVRSSARQT---LKAAPLADQARLDEMYDHLDKHGDGVKDVAFEVDDV
Hordeum vulgare                 TAPYANGCDAA------TASLPSFSADAARRFSADHGIAVRSVALRVADA
Zea mais                        TAPYAHGADAA------TAALPSFSAAAARRFAADHGLAVRAVALRVADA
Arabidopsis thaliana            TAPYSPSLSAGSIKPTTTASIPSFDHGSCRSFFSSHGLGVRAVAIEVEDA
Daucus carota                   TAPYSPSTTTSSGS----AAIPSFSASGFHSFAAEHGLAVRAIALEVADV
Streptomyces avermitilis        TSVIK--P--A---TPWG--------SFLADHVAEHGDGVVDLAIEVPDA
Pseudomonas fluorescens         NNEPH----------S---------VASYFAAEHGPSVCGMAFRVKDS
                                                .  ** .*  :*:.*

                                !                   !         !
                                60                  70        80
```

FIG 1

```
Mus musculus                    DHIVQKARERGAKIVREPWVEQDKPGKVKFAVLQTYGDTTHTLVEKINYT
Coccidioides immitis            ESVFSAAVRNGAKVVSDVRTVKDEDGQIKMATIRTYGSTTHTLIERSGYR
Mycosphaerella graminicola      LAVYENAVANGARSVSSPHTDSCDEGDVISAAIKTYGDTTNTFIQRTTYT
Hordeum vulgare                 ABAFRASRRRGARPAFAPVDLGRG---FAPAEVELYGDVVLREVSEPDGT
Zea mais                        EDAFRASVAAGARPAFGPVDLGRG---FRLAEVELYGDVVLRYVSYPDGA
Arabidopsis thaliana            ESAFSISVANGAIPSSPPIVLNEA---VTIAEVKLYGDVVLRYVSYKAED
Daucus carota                   AAAFEASVARGARPASAPVELDDQ---AWLAEVELYGDVVLRFVSFGREE
Streptomyces avermitilis        RAAHAYAIEHGARSVAEFYELKDEHGTVVLAAIATYGKTRHTLVDRTGYD
Pseudomonas fluorescens         QKAYKRALELGAQPIEIETGPMKLN----LPAIKGIQGAPLYLIDRFGEG
                                      :  **                .  :   *  .    :.

P. fluorescens numbering        !        !        !             !        !
                                90       100      110           120      130

Mus musculus                    G---RFLPGFEAPTYKDTLLPKLPRCNLEIIDHIVGNQFDQEMQSASEWY
Coccidioides immitis            G---GFMPGYEMESNADATSKFLPKVVLERIDECVGNQDWDEMERVCDYY
Mycosphaerella graminicola      G---PFLPGYRSCTTVDSANKFLPPVNLEAIDECVGNQDWDEMSDACDFY
Hordeum vulgare                 D--VPFLPGFEQVTNPDAVD-----YGLTRFDHVVGNVP--ELAPAAAYI
Zea mais                        AG-EPFLPGFEGVASPGAAD-----YGLSRFDHIVGNVP--ELAPAAAYP
Arabidopsis thaliana            TEKSEPFLPGFERVEDASSFP-LD--YGIRRLDHAVGNVP--ELGPALTYV
Daucus carota                   G---LFLPGFEAVEGTASFPDLD--YGIRRLDHAVGNVT--ELGPVVEYI
Streptomyces avermitilis        G---PYLPGYVAAA---PIVEPPAHRTPQAIDHCVGNVELGRMNEWVGFY
Pseudomonas fluorescens         S--SIYDIDPVFLEGVDREP-VG--AGLKIIDHLTENVYEGRMAYWANFY
                                     :  .:                 :  :** . *    .:     :

P. fluorescens numbering        !        !        !        !
                                140      150      160      170

Mus musculus                    LKNLQFERFWSVDDTQVETEYSSLRSIVVTNYEESIKMPINEPAPG-RKK
Coccidioides immitis            EKILGFERFWSVDDKDICTEYSALKSIVMASFNDIVKMPINEPAKG-KKQ
Mycosphaerella graminicola      ERCLGFERFWSVDDKDICTEFSALKSIVMSSFNQVVKMPINEPAHG-KKK
Hordeum vulgare                 AGFTGFHEFAEFTAEDVGTTESGLNSVVLANNSEGVLLPLNEPVHGTKRR
Zea mais                        AGPTGFHEFAEFTTEDVGTAESGLNSMVLANNSENVLLPLNEPVHGTKRR
Arabidopsis thaliana            AGFTGFEQFAEFTADDVGTAESGLNSAVLASNDEMVLLPINEPVHGTKRK
Daucus carota                   KGFTGFEEFAEFTAEDVGTLESGLNSVVLANNEEMVLLPLNEPVYGTKRK
Streptomyces avermitilis        NKVMGFTNMKEFVGDDIATRYSALMSKVVADGTLKVKFPINEPALA-KKK
Pseudomonas fluorescens         EKLFNFRSIRYF---DIKGEYTGLTSKAMTAPDGMIRIPLNEESSK--GA
                                     *  .:  .   ::    :.* * .::      : :*:**

P. fluorescens numbering        !        !        !        !        !
                                180      190      200      210      220
```

FIG 1 (continuation)

```
Mus musculus                    SQIQEYVDYNGGAGVQHIALKTEDIITAIRHLRER----GTEFLAAP-SS
Coccidioides immitis            SQIEEYVDFYNGAGVQHIALRTNNIIDAITNLKAR----GTEFIKVP-ET
Mycosphaerella graminicola      SQIEEYVDFYNGPGVQHIALRTPNIIEAVSNLRSR----GVEFISVP-DT
Hordeum vulgare                 SQIQTFLEHHGGPGVQHIAVASSDVLRTLRKMRARSAMGGFDFLPPPLPK
Zea mais                        SQIQTFLDHHGGPGVQHMALASDDVLRTLREMQARSAMGGFEFMAPPTSD
Arabidopsis thaliana            SQIQTYLEHNEGAGLQHLALMSEDIFRTLREMRKRSSIGGFDFMPSPPPT
Daucus carota                   SQIQTYLEHNEGAGVQHLALVSEDIFRTLREMEKRSCLGGFEFMPSPPPT
Streptomyces avermitilis        SQIDEYLEFYGGAGVQHIALNTGDIVETVRTMRAA----GVQFLDTP-DS
Pseudomonas fluorescens         GQIEEFLMQFNGEGIQHVAFLSDDLIKTWDHLRSI----GMRFMTAPPDT
                                .**: ::     * *:**:*. : ::. :   ::       *  *:  *

P. fluorescens numbering        |         |         |         |         |
                                230       240       250       260       270

Mus musculus                    YYKLLRENLKSAKIQVKESMDVLEELEILVD-YDEKG---YLLQIFTKPM
Coccidioides immitis            YYEDMKIRLKRQGLVLDEDFETLKSLDILID-FDENG---YLLQLFTKHL
Mycosphaerella graminicola      YYENMRLRLKAAGMRLEESFDIIQKLNILID-FDEGG---YLLQLFTKPL
Hordeum vulgare                 YYEGVRRLAG--DVLSEAQIKECQELGVLVD-RDDQG---VLLQIFTKPV
Zea mais                        YYDGVRRRAG--DVLTEAQIKECQELGVLVD-RDDQG---VLLQIFTKPV
Arabidopsis thaliana            YYQNLKKRVG--DVLSDDQIKECEELGILVD-RDDQG---TLLQIFTKPL
Daucus carota                   YYKNLKNRVG--DVLSDEQIKECEDLGILVD-RDDQG---TLLQIFTKPV
Streptomyces avermitilis        YYDTLGERVG----DTRVPVDTLRELKILAD-RDEDG---YLLQIFTKPV
Pseudomonas fluorescens         YYEMLEGRLP----NHGEPVGRLQARGILLDGSSESGDKRLLLQIFSHTL
                                **. :                .   .   :* *  .: *    ***:*:: :

P. fluorescens numbering        |         |         |         |
                                280       290       300       310

Mus musculus                    QDRPTLFLEVIQR---------H------NHQGFGAGNFNSLFKAFEE-E
Coccidioides immitis            MDRPTVFIEIIQR---------N------NFSGFGAGNFRALFEAIER-E
Mycosphaerella graminicola      MDRPTVFIEIIQR---------N------NFDGFGAGNFKSLFEAIER-E
Hordeum vulgare                 GDRPTLFLEMIQRIGCMEKDERGEHYQKGGCGGFGKGNFSELFKSIEDYE
Zea mais                        GDRPTLFLEIIQRIGCMEKDEKGQEYQKGGCGGFGKGNFSQLFKSIEDYE
Arabidopsis thaliana            GDRPTIFIEIIQRVGCMEKDEEGKAYQSGGCGGFGKGNFSELFKSIEEYE
Daucus carota                   GDRPTLFIEIIQRVGCMLKDDAGQMYQKGGCGGFGKGNFSELFKSIEEYE
Streptomyces avermitilis        QDRPTVFFEIIER---------H------GSMGFGKGNFKALFEAIER-E
Pseudomonas fluorescens         MG--PVFFEFIQR---------K------GDDGFGEGNFKALFESIER-D
                                .  .:*:*.*:*                  .  *** *** **:::*  :

P. fluorescens numbering        |         |         |
                                320       330       340
```

FIG 1 (continuation)

```
Mus musculus                    QALRGNLTDLEPNGVRSGM
Coccidioides immitis            QALRGTLI----------
Mycosphaerella graminicola      QDLRGNL-----------
Hordeum vulgare                 KSLEAKQS---AAVQGS--
Zea mais                        KSLEAKQAAAAAAAQGS--
Arabidopsis thaliana            KTLEAKQLVG--------
Daucus carota                   KTLEAKQITGSAAA-----
Streptomyces avermitilis        QEKRGNL-----------
Pseudomonas fluorescens         QVRRGVLSTD--------
                                :  ..

P. fluorescens numbering         1
                                350
```

FIG 1 (end)

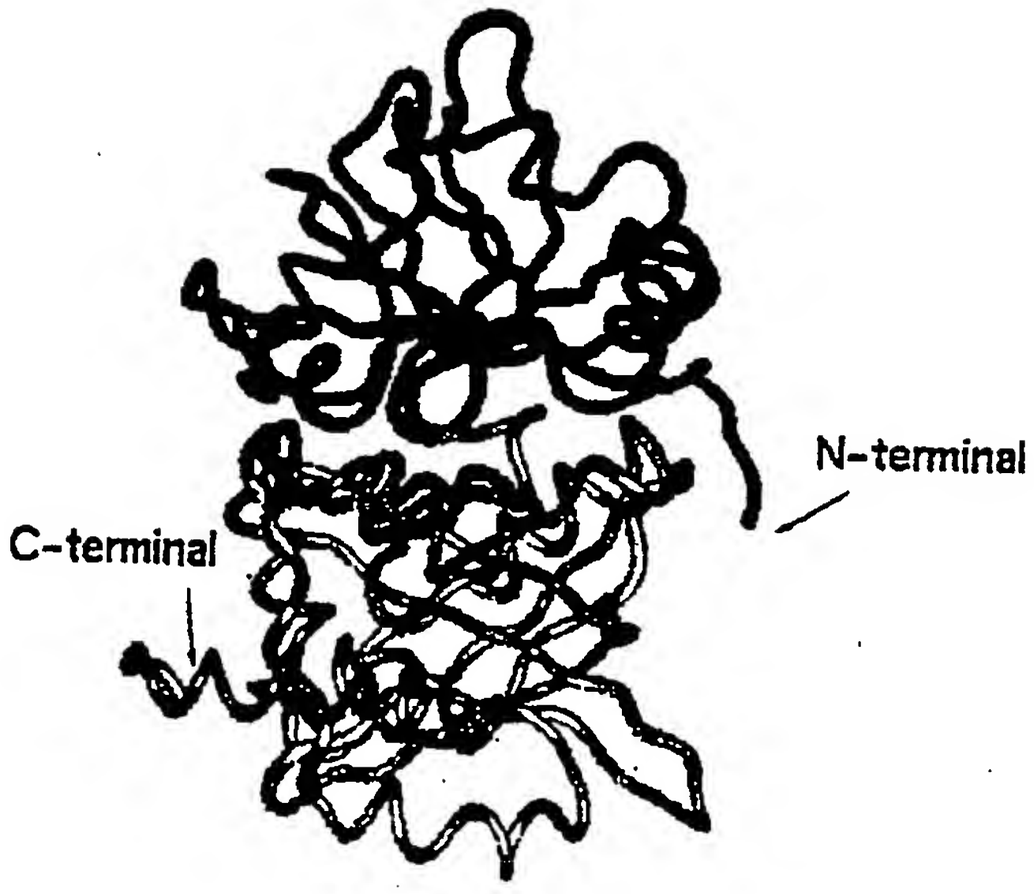

FIG 2

Seq_1: Pseudomonas fluorescens (bacterium) HPPD 357 aa

Seq_2: Synechocystis (cyanobacterium) HPPD 339 aa

```
Seq_1MADLYENPMGL MGFEFIELAS PTPNTLEPIP Q.IMGFTKVA THRSND..VH
Seq_2            MEFDYLHLYV EDYQSAHRCY QRQWGFTCVN KIITDQGITG

Seq_1 LYRQGAINLI LNEEPHSVAS Y..FAAEHGP SVCGMAFRVK DSQKAYKRAL
Seq_2 IYQQGQILLL ISASESSLSR YADYLQKHPP GVGEVAWQVA NWQKIQHQLS

Seq_1 ELGAQPIHIE TGPMELNLPA IKGIGGAPLY LIDRFGEGSS IYDIDFVFLE
Seq_2 EL....Q.IE TTPVIH..PL TKAEGLTFLL WGDVH...HS IYPVRSELNQ

Seq_1 GVDRHPVGAG LKIIDHLTHN VYRGRMAYWA NFYEKLFNFR EIRYFDIKGE
Seq_2 NKTLH..GVG LTTIDHVVLN LAADQFTQAS QWYQQVFGWS VQQSFTVNTP

Seq_1 YTGLTSKAMT APDGMIRIPL NEESSKGAGQ IEEFLMQFNG EGIQHVAFLS
Seq_2 HSGLYSEALA SANGKVQFNL NCPTNN.SSQ IQTFLANNHG AGIQHVAFST

Seq_1 DDLIKTWDHL KSIGMRFMTA PPDTYYEMLE GRLPNHG.EP VGELQARGIL
Seq_2 TSITRTVAHL RERGVNFLKI PTGYYQQQRN SSYFNYASLD WDTLQCLEIL

Seq_1 LDGSSESGDK RLLLQIFSET LMGP..VFFE FIQRKG.DDG FGEGNFKALF
Seq_2 LDDQENTG.E RLLLQIFSQP CYGVGTLFWE IIERRHRAKG FGQGNFQALY
                                                  * *
Seq_1 ESIERDQVRR GVLSTD
Seq_2 EAVETLEKQL EVP..
```

FIG 3